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(54) Title: FUNCTIONALIZED DERIVATIVES OF HYALURONIC ACID AND FORMATION OF HYDROGELS IN SITU USING SAME

(57) Abstract

Methods for chemical modification of hyaluronic acid, formation of amine or aldehyde functionalized hyaluronic acid, and the cross–linking thereof to form hydrogels are provided. Functionalized hyaluronic acid hydrogels of this invention can be polymerized *in situ*, are biodegradable, and can serve as a tissue adhesive, a tissue separator, a drug delivery system, a matrix for cell cultures, and a temporary scaffold for tissue regeneration.

FUNCTIONALIZED DERIVATIVES OF HYALURONIC ACID AND FORMATION OF HYDROGELS IN SITU USING SAME

TECHNICAL FIELD OF THE INVENTION

This invention is directed to biomaterials for spatially and temporally controlled delivery of bioactive agents such as drugs, growth factors, cytokines or cells. In particular, this invention teaches versatile methods for chemical crosslinking of high molecular weight hyaluronic acid under physiological conditions *in situ*, to form polymerizable biodegradable materials. The methods are based on the introduction of functional groups into hyaluronic acid (HA) via formation of an active ester at the carboxylate of the glucuronic acid moiety as an intermediate and subsequent substitution with a side chain containing a nucleophilic group on one end and a (protected) functional group on the other end. The introduced functional groups allow for crosslinking of the HA derivatives. Crosslinked hyaluronic acid hydrogels of this invention are useful in various surgical applications and as a temporary scaffold for tissue regeneration, e.g., in cartilage repair.

BACKGROUND OF THE INVENTION

Repair of articular cartilage

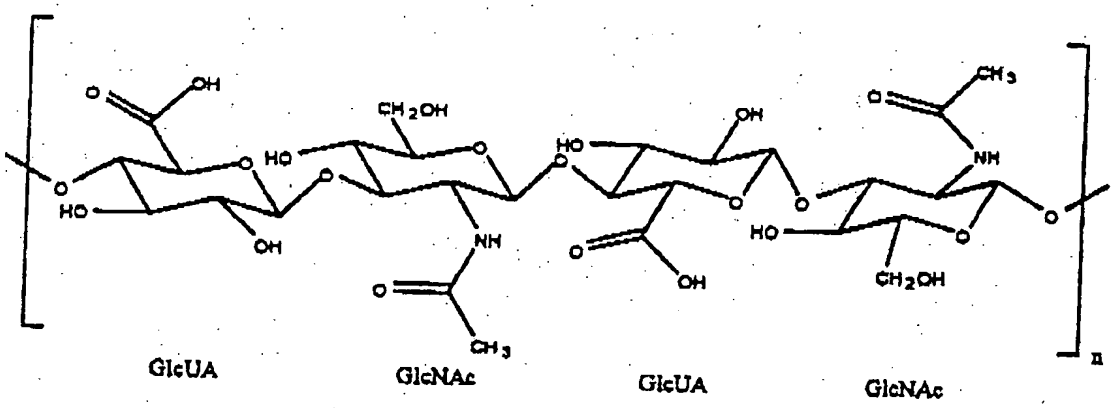
The failure of regenerating persistent hyaline cartilage by surgical procedures prompted investigators to attempt repair using biological strategies.

of candidate matrix materials has been the limiting factor, and anchoring of materials seeded with chondrocytes and/or chondrogenic factors difficult, explaining the unsatisfactory results obtained with currently available materials such as polylactic acid and polyglycolic acid scaffolds (Freed et al., J. Biomed. Mat. Res. 28, 891-899 (1994); Chu et al., J. Biomed. Mat. Res. 29, 1147-1154 (1995)), calcium phosphate minerals (Nakahara et al., Clin. Orthrop. 276, 291-298 (1992)), fibrin sealants (Itay et al., Clin. Orthrop. 220, 284-303 (1987)), and collagen gels (Wakitani et al., J. Bone Joint Surg. 71-B, 74-80 (1989)). We have developed novel biodegradable materials based on hyaluronic acid which are optimized for the biological requirements posed on a repair material in a synovial joint and allow *in situ* polymerization.

Biology of hyaluronic acid and its therapeutic use

Hyaluronic acid (HA) is unique among glycosaminoglycans in that it is not covalently bound to a polypeptide. HA is also unique in having a relatively simple structure of repeating nonsulfated disaccharide units composed of D-glucuronic acid (GlcUA) and *N*-acetyl-D-glucosamine (GlcNAc) (Scott et al., The Chemistry, Biology and Medical Applications of Hyaluronan and Its Derivatives, T.C. Laurent (ed.), Portland Press, London, (hereinafter "Hyaluronan and Its Derivatives"), pp. 7-15 (1998)). Its molecular mass is typically several million Daltons. HA is also referred to as hyaluronan or hyaluronate, and exists in several salt forms (see formula I).

(I)



Its Derivatives, pp. 33-42 (1998)). Extensive efforts have been made by various laboratories to produce derivatives of HA with unique properties for specific biomedical applications. Most of the developments have been focusing on the production of materials such as films or sponges for implantation and the substitution of HA with therapeutic agents for delayed release and/or prolonged effect (for review see Band, supra; Prestwich et al., Hyaluronan and Its Derivatives, pp. 43-65 (1998); Gustafson, Hyaluronan and Its Derivatives, pp. 291-304 (1998)). Strategies have included esterification of HA (U.S. Patent Nos. 4,957,744 and 5,336,767), acrylation of HA (U.S. Patent No. 5,410,016), and cross-linking of HA using divinyl sulfone (U.S. Patent No. 4,582,865) or glycidyl ether (U.S. Patent No. 4,713,448). However, the modified HA molecules show altered physical characteristics such as decreased solubility in water and/or the chemical reaction strategies used are not designed for crosslinking of HA under physiological conditions (in an aqueous environment, at pH 6.5-8.0).

It is well known that polyaldehydes can be generated by oxidizing sugars using periodate (Wong, CRC Press, Inc., Boca Rayton, FL, pp. 27 (1993); European Patent No. 9615888). Periodate treatment oxidizes the proximal hydroxyl groups (at C2 and C3 carbons of glucuronic acid moiety) to aldehydes thereby opening the sugar ring to form a linear chain (Scheme 1). While periodate oxidation allows for the formation of a large number of functional groups, the disadvantage is the loss of the native backbone structure. Consequently, the generated derivative may not be recognized as HA by cells. In fact hydrogels formed by using periodate oxidized HA as a crosslinker, e.g., in combination with the HA-amines described herein, showed very limited tissue transformation and poor cellular infiltration in the rat ectopic bone formation model (Fig. 6). This is in sharp contrast to the HA-aldehyde derivatives described herein.

The introduction of free amino groups on HA, which could be used for further convenient coupling reactions under mild physiological conditions, has been a subject of great interest. Previous methods have produced a free amino group on high molecular weight HA by alkaline *N*-deacylation of its glucosamine

HA. It is a further objective that the method of functionalization provides HA molecules that are well tolerated *in vivo* and are biodegradable.

It is also the objective of this invention to identify HA derivatives and methodology for *in situ* polymerization thereof to provide a biodegradable scaffold for tissue regeneration. It is another objective that the HA materials can be polymerized in the presence of cells to serve as a vehicle for cell transplantation. It is a further objective to provide methodology for functionalization and cross-linking of HA that allows for variations in the biomechanical properties of the formed gels as well as in the sensitivity to cellular infiltration and degradation.

SUMMARY OF THE INVENTION

Biomaterials for spatially and temporally controlled delivery of bioactive agents such as drugs, growth factors, cytokines or cells, are a key factor for tissue repair. In particular, *in situ* polymerizable biodegradable materials are needed for cartilage resurfacing that are designed to withstand the mechanical forces in a joint. We have developed a versatile method for chemical crosslinking of high molecular weight hyaluronic acid under physiological conditions. The method is based on the introduction of functional groups into hyaluronic acid by formation of an active ester at the carboxylate of the glucuronic acid moiety and subsequent substitution with a side chain containing a nucleophilic group on one end and a (protected) functional group on the other end. We have formed hyaluronic acid with amino or aldehyde functionality, and formed hydrogels with modified hyaluronic acid and bifunctional crosslinkers or mixtures of hyaluronic acid carrying different functionalities using active ester- or aldehyde-mediated reactions. Physical and chemical properties of the hydrogels of this invention were evaluated using biomechanical testing, and by assaying sensitivity towards degradation by glycosidases such as testicular hyaluronidase. Biocompatibility was evaluated using cell culture assays and subcutaneous implantation of the hyaluronic acid materials in rats. This *in vivo* assay is also the established model for induction of ectopic bone formation by members of the transforming growth factor β family (TGF-β), and

of functional groups, for crosslinking to generate hydrogels with excellent physical properties. HA hydrogels have also successfully been used as a delivery vehicle in chondrocyte transplantation studies (Robinson et al., Calcif. Tissue Int. 46, 246-253 (1990)) and HA has proven its biocompatibility in various forms in clinical practice (for review see Laurent and Fraser, supra; Balazs and Laurent, supra).

The reaction mechanisms we have explored for *in situ* polymerization of HA derivatives are compatible with an aqueous environment and are non-toxic to cells. The aldehyde-mediated crosslinking strategies follow reactions occurring physiologically in crosslinking of fibrillar collagens and elastin. NHS-esters provide an alternative for rapid formation of stable bonds under physiological conditions, primarily by reaction with primary amines. The technology of NHS-ester-mediated protein crosslinking has been developed for materials with applications in plastic surgery that require *in situ* polymerization (U.S. Patent No. 5,413,791).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results of a ninhydrin test after reductive alkylation of HA and HA-aldehyde in the presence of putrescine. Reductive alkylation was carried out with an excess of putrescine in the presence of pyridine borane. HA or derivatives thereof were purified by repeated ethanol precipitation prior to detection of free amino groups on the HA chain by using the ninhydrin test (Sheng et al., Anal. Biochem. 211, 242-249 (1993)).

Fig. 2 shows ^{1}H NMR of native HA (Fig. 2A) and an HA-derivative with protected aldehyde functionality (Fig 2B) in D_2O at 300 MHz. Peaks are assigned as indicated on the structural formula.

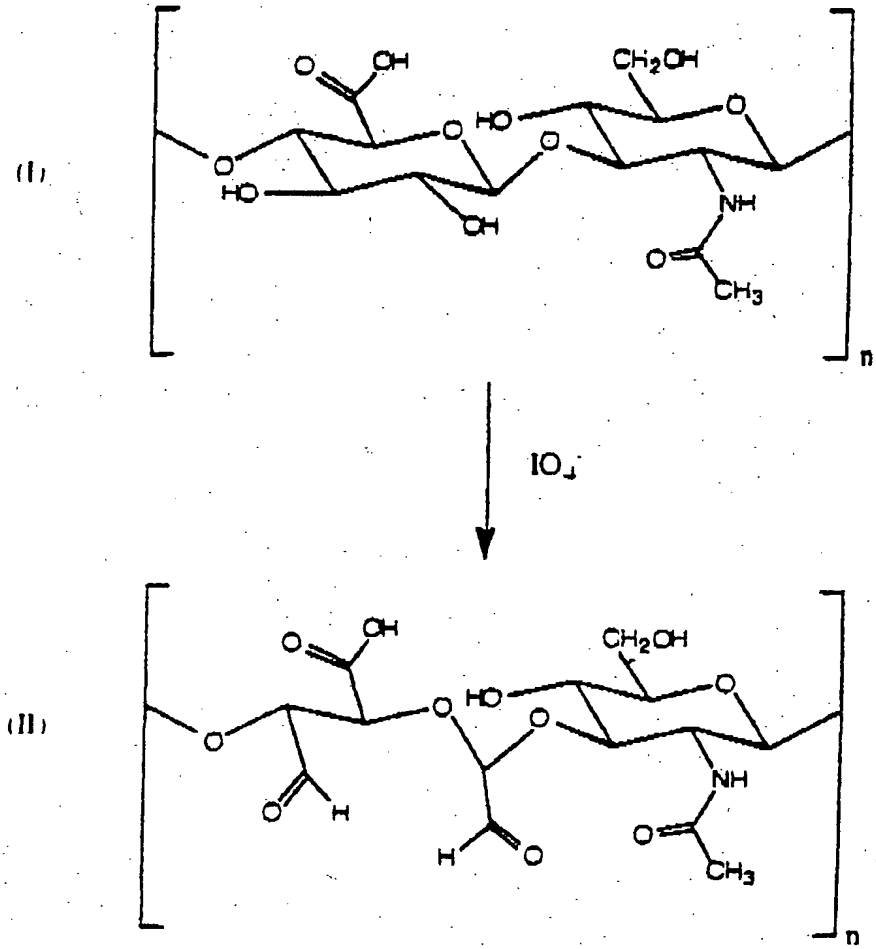
Fig. 3 shows ^{1}H NMR of HA-derivatives with amine functionality formed from putrescine (Fig. 3A), histidine (Fig. 3B), lysine (Fig. 3C), and adipic dihydrazide (Fig. 3D) in D_2O at 300Mhz. Peaks are assigned as indicated on the structural formula.

modified (~20-25%) HA-amine (adipic dihydrazido-HA) crosslinked with 0.25 mg/ml glutaraldehyde, (Fig. 6B) 7 mg/ml of the same HA-amine crosslinked with 7 mg/ml HA-aldehyde (periodate oxidized), (Fig. 6C) 7 mg/ml of the same HA-amine crosslinked with 7 mg/ml HA-aldehyde (deprotected amino-dimethyl acetal-HA, ~10-15% modified), or (Fig. 6D) 7 mg/ml of the same HA-amine crosslinked with 7 mg/ml HA-aldehyde (deprotected hydrazido-dimethyl acetal-HA, 40-45% modified). The hydrogels also contained 1 mg/ml prefibrillized intact collagen type I, 200 µg/ml BMP-2 and 500 ng/ml IGF-1 to induce bone formation. Tissue specimens were harvested 10 days post implantation, fixed in formalin and processed for histology by paraffin embedding. Sections were stained with Haematoxylin/Eosin. m, matrix material (note: matrix material shrinks during dehydration); s, skin (indicates orientation of implant).

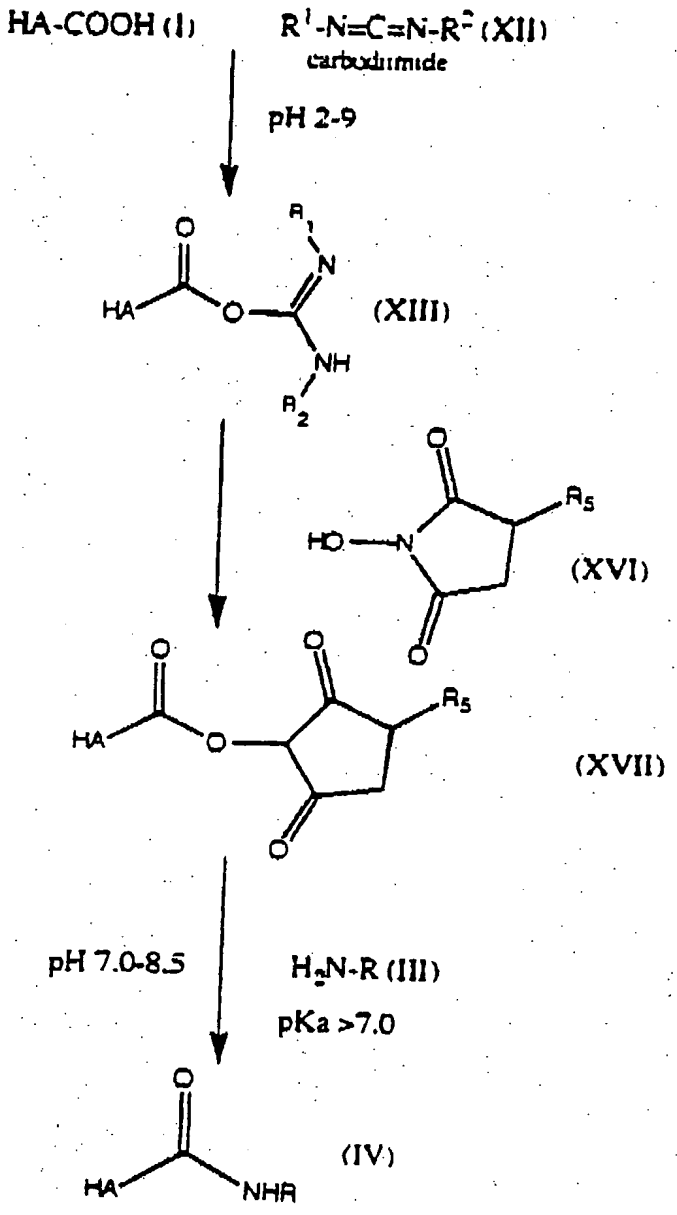
Fig. 7 shows *in vivo* evaluation of HA hydrogels crosslinked with different NHS-esters. Subcutaneous implantation in rats of HA hydrogels consisting of (Fig. 7A) 12 mg/ml highly modified (~65-70%) HA-amine (adipic dihydrazido-HA) crosslinked with 15 mg/ml $(SPA)_2$-PEG, (Fig. 7B) 12 mg/ml optimally modified (~20-25%) HA-amine (adipic dihydrazido-HA) crosslinked with 15 mg/ml SPA_2-PEG, or (Fig. 7C) 12 mg/ml of the same optimally modified HA-amine crosslinked with 3 mg/ml DTSSP (crosslinker concentrations are equal on a molar basis). The hydrogels also contained 1 mg/ml prefibrillized intact collagen type I, 200 µg/ml BMP-2 and 50 ng/ml TGF-β2 to induce bone formation. Tissue specimens were harvested 10 days post implantation, fixed in formalin and processed for histology by paraffin embedding. Sections were stained with Haematoxylin/Eosin. m, matrix material (note: matrix material shrinks during dehydration); s, skin (indicates orientation of implant).

Fig. 8 shows differential effect of growth factors incorporated into HA hydrogels on tissue transformation. Subcutaneous implantation in rats of the HA hydrogel formed from 12 mg/ml optimally modified (~20-25%) HA-amine (adipic dihydrazido-HA) crosslinked with 15 mg/ml $(SPA)_2$-PEG. The hydrogels also contained 1 mg/ml prefibrillized intact collagen type I, and were supplemented

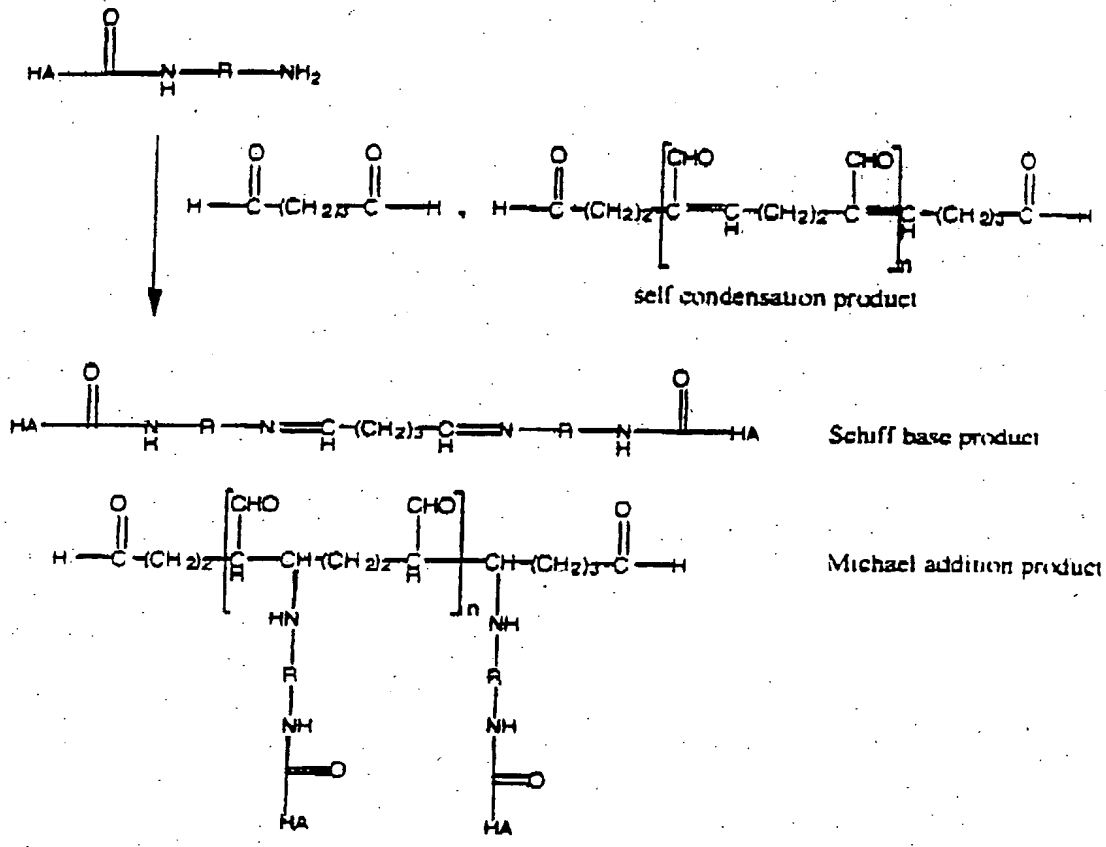
Scheme I



Scheme 3


HA-COOH (I)
R1-N=C=N-R2 (XII)
carbodiimide
pH 2-9
(XIII)
(XVI)
(XVII)
pH 7.0-8.5
H2N-R (III)
pKa >7.0
(IV)

Scheme 5


self condensation product
Schiff base product
Michael addition product

DETAILED DESCRIPTION OF THE INVENTION

Using the methods of our invention, we generate an activated form of HA that differs minimally from native HA to conserve its unique physico-chemical properties. We also effect a minimal change affecting only a relatively small number of dissaccharide units of native HA so that we do not alter its property to serve as a cell substratum.

We initially attempted to generate an aldehyde derivative of HA by reduction of the carboxyl groups of the glucuronic acid moieties into aldehydes using 9-borabicyclo-3,3-nonane, a method that allows direct conversion of the carboxylic acid into the aldehyde (Cha et al., Bull Korean Chem. Soc. 9, 48-52 (1988), Cha et al., Org. Prep. Proc. Int. 21, 451-477 (1989)):

1) HA-COOH (I) ------> HA-CHO (II)

However, even though preliminary testing indicated the conversion of the carboxyl groups into aldehydes to a degree of approximately 5-10% (Fig. 1), mixtures of concentrated, viscous HA-aldehyde solutions (~10 mg/ml) with 'small' polyamines such as putrescine, lysine, polylysine, histidine, or polyhistidine did not generate stable gels in a reasonable time frame to be suitable for *in situ* polymerization. It is important to note that the chemical properties of HA are determined not merely by its functional groups *per se* but also by the accessibility of these functional groups of HA in an aqueous environment, which is related to the overall conformational structure and rheological properties of HA. HA behaves like a hydrogel in an aqueous media even in the absence of crosslinks because it forms a network stabilized by hydrogen bonds and van der Waals forces (Laurent and Fraser, supra). To increase the accessibility of functional groups, we introduced a spacer between the functional group and the HA chain.

Introducing a functionalized side chain onto HA

We have subsequently developed methodology for introducing side chains into HA by carbodiimide-mediated coupling of primary or secondary amines

for the coupling reaction to be carried out at neutral pH (about 7.0 to 8.5) and consequently yields products by reaction with simple primary amines (Scheme 3).

Consequently, this methodology allows for the following reactions to be carried out:

HA-COOH (I) + H_2N-R (III) -----> HA-CO-NH-R (IV)

HA-COOH (I) + R'-NH-R (V) -----> HA-CO-NR'-R (VI)

wherein R and R' are alkyl, aryl, alkylaryl or arylalkyl side chains which may contain hetero atoms such as oxygen, nitrogen, and sulfur. The side chain may be branched or unbranched, and be saturated or may contain one or more multiple bonds. The carbon atoms of the side chain may be continuous or may be separated by one or more functional groups such as an oxygen atom, a keto group, an amino group, and oxycarbonyl group, etc. The side chain may be substituted with aryl moieties or halogen atoms, or may in whole or in part be formed by ring structures such as cyclopentyl, cyclohexyl, cycloheptyl, etc. The side chain may have a terminal functional group for crosslinking such as aldehyde, amine, arylazide, hydrazide, maleimide, sulfhydryl, etc. The side chain may be a bioactive peptide, e.g., containing receptor binding sites or proteolytic cleavage sites.

Terminal functional groups of the side chain useful for crosslinking of HA under physiological conditions may be selected from the following list:

1. aldehydes, see examples

(VII) H_2N-R-CHO

2. amines, see Examples

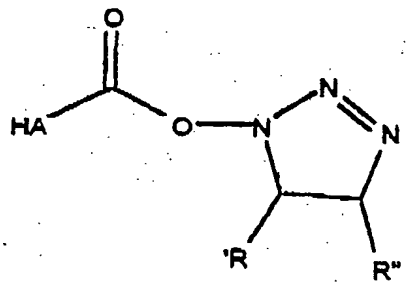
(VIII) H_2N-R-NH_2

3. arylazides, e.g., 4-(*p*-azidosalicylamido) butylamine

The active ester may be of the following class and be formed by carbodiimide-mediated coupling of a compound for preparation of these active esters known to a person in the art:

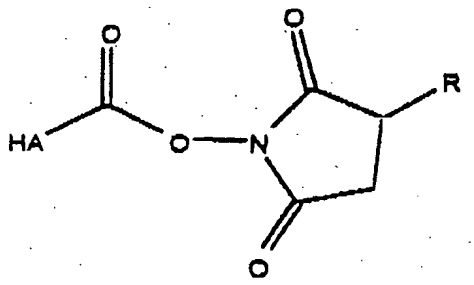
1. triazole esters, *e.g.* 1-hydroxybenzotriazole

(XIII)



2. NHS-esters, *e.g.* *N*-hydroxysulfosuccinimide

(XIV)

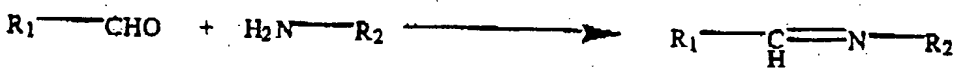


~6.5 by repeated addition of 0.1M HCl during the reaction (Scheme 2). HA-derivatives were also prepared in a similar manner using *N*-hydroxy-sulfosuccinimide and primary amines containing unconjugated amino groups with a higher pKa (>9) such as 1,4-diaminobutane or 1,6 diaminohexane (Scheme 3). The HA derivatives were purified by repeated ethanol precipitation or by extensive dialysis, and the nature of the HA derivatives was confirmed by ^{1}H NMR (Fig. 3). The degree of modification was calculated from the NMR data and found to be as high as 70%. Reaction conditions were subsequently adjusted such that a degree of modification of approximately 20% was achieved. Limiting the amount of carbodiimide proved to be most successful in controlling the degree of modification. A degree of modification of 10-25% yielded efficient crosslinking but also a molecule that would still be recognized by glycosidases and by HA receptors as HA and thus allow for recognition and processing of the material by cells (see below). Similar HA derivatives were synthesized using succinic, adipic or suberic dihydrazide or diaminoethane, -butane, or -hexane to study the effect of the length of the spacer separating the introduced functional group from the HA-chain on the subsequent crosslinking. See Examples 4-8.

Crosslinked HA hydrogels

The functionalized HA molecules can be crosslinked by reacting HA derivatives with different functionalities or using homo- or heterobifunctional crosslinkers which are available in large variety. The following basic reaction schemes are suitable for crosslinking of the described forms of modified HA (see Examples 9-12):

1. aldehyde-mediated crosslinking



2. active ester-mediated crosslinking

derivative with >0.05 mM aldehyde or >0.2 mM active ester (numbers are reflecting functional group concentrations). Optimal gels were generated by crosslinking 10-15mg/ml HA derivative, modified to a degree of about 10-25%, with about 0.2mM aldehyde or 0.6mM active ester. Similarly, crosslinking of the HA-aldehyde derivatives (Mr ~10^4) (optimally about 10-15mg/ml) with bifunctional amines (optimally about 0.2mM) yielded excellent gels (Scheme 6). Conjugated amines such as dihydrazines or benzylamines are required for *in situ* polymerization of HA in this case to resonance stabilize the unstable Schiff base product formed by reaction of an aldehyde with a primary amine (i.e., hydrazines yield a more stable hydrazone linkage). Hydrogels were also formed from an equimolar mixture of HA-aldehyde derivatives and the different HA-amine derivatives (Scheme 6). Optimal gels were formed with ~15mg/ml of the HA derivatives. At the optimal concentrations of HA and crosslinker, gelation occurred typically in about 30 sec. to 5 min. which is suitable for *in situ* polymerization. The crosslinked HA hydrogels were sensitive to glycosidases, i.e., testicular hyaluronidase, indicating that they are biodegradable (Fig. 4).

A number of different tests including cell culture assays and animal experiments served to assess biocompatibility of the formulated biomaterials. Embedding of chondrocytes into the polymerizing HA hydrogels showed that neither aldehyde nor NHS-ester-mediated crosslinking was toxic to cells at the concentrations employed. Seeding of cells on top of prepolymerized HA hydrogels showed a wide variety of cellular behaviours depending on the nature of the crosslinker and crosslinking density (Fig. 5). Highly crosslinked HA hydrogels were impenetrable to cells (Fig. 5, A and B), while optimally crosslinked gels were infiltrated (Fig. 5C). Supplementation of the HA hydrogels with cell adhesion molecules such as fibronectin (in the range of 0.1 to 1 mg/ml) did induce adhesion and spreading of cells on the materials independent of the nature of the crosslinker and the crosslinking density, but did not change the results with regard to cell infiltration, demonstrating that the lack of infiltration is due to the high crosslinking density and not the absence of cell-matrix interactions. See below and Fig. 7.

effect of TGF-β2 (Yang and Moses, J. Cell. Biol., 111, 731-741 (1990)). This demonstrates that the biological activity of the HA material can be modulated by inclusion of different bioactive factors. The lack of significant adverse effects and the demonstration of the desired biological activity of these novel HA biomaterials *in vivo* demonstrates their usefulness as a delivery vehicle for cells and growth factors in the field of tissue regeneration.

There are several approaches to the production of HA, including extraction from tissue and biosynthesis. Extraction from tissue typically uses fresh or frozen cocks' combs (U.S. Pat. No. 5,336,767), although other tissues including the synovial fluid of joints (Kvam et al., Anal. Biochem. 211, 44-49 (1993)), human umbilical cord tissue, bovine vitreous humor, and bovine tracheae, have been used. It is also possible to prepare HA by microbiological methods, such as by cultivating a microorganism belonging to the genus Streptococcus which is anhemolytic and capable of producing HA in a culture medium (U.S. Pat. Nos. 4,897,349; 4,801,539; 4,780,414; 4,517,295; 5,316,926). The HA raw material for preparing the compositions of the invention preferably consists of high molecular weight HA, more preferably of molecular weight greater than 0. 5 million daltons, and more preferably of molecular weight greater than one million daltons. The HA raw material for the compositions of examples of this invention described herein was obtained from Genzyme Corp. (Cambridge, MA), and had a molecular weight greater than one million daltons. The size of the HA was unchanged after derivatization.

The compositions of the invention have many therapeutic uses. The fact that the compositions may be cured in a surgically practical time frame of one to five minutes *in situ* with concurrent crosslinking to the tissue surfaces allows for employment as a tissue glue. Many situations in various surgical applications require such adhesives. For example, the compositions of the invention may be used to stem hemorrhage in general surgery, reconstruct nerves and vessels in reconstructive, neuro- and plastic surgery, and to anchor skin, vascular, or cartilage transplants or grafts in orthopedic, vascular, and plastic surgery. Those of skill in

many situations. For example, the compositions of the invention may be used not only to promote bone formation and stimulate cartilage repair in orthopedic procedures, as described more fully below, but also to treat pathological wound conditions such as chronic ulcers. They may also serve as a scaffold to generate artificial tissues, e.g., cartilage (Hauselmann et al., Am. J. Physiol. 271, C742-752 (1996)), through proliferation of autologous cells in culture. Similar procedures for generation of equivalents of other tissues or organs, including skin, liver, and others, in culture may be developed in the future and may be used in combination with the compositions of the invention.

Highly crosslinked materials have an anti-adhesive property with respect to cells, and such compositions may be used to generate tissue separations and to prevent adhesions following surgery. See Figs. 5A and 7C, showing highly modified HA-amine, i.e., adipic dihydrazido HA, preferably crosslinked with low molecular weight bifunctional NHS-ester. The viscoelastic properties of HA make it particularly well suited for this purpose, and it is used clinically to achieve temporal pain relief by repeated intraarticular injections in arthropathies as a "joint lubricant", and as a protective agent for eye irritations and in ophthalmic surgery. The technique of tissue separation is used in facial reconstruction in plastic surgery and dentistry. Prevention of the formation of adhesions is particularly relevant in reconstructive surgery of tendons, in surgical procedures in the urogenital system, and in thoracic surgery. Many different HA-based materials are already in clinical use in these areas. (See products manufactured by Anika Therapeutics, Inc. (Woburn, MA), Biomatrix, Inc. (Ridgefield, NJ), Genzyme Corp. (Cambridge, MA), and Fidia, S.p.A. (Abano Terme, Italy)). Those of skill in the art may choose and design particular embodiments of the invention which are particularly suitable for a desired application by selecting distinct features as outlined above.

The injectable nature of the compositions of the invention also renders them suitable for tissue augmentation in plastic surgery, where the HA matrix serves primarily as a biocompatible filler material, e.g., for filling dermal creases or lip reconstruction. Again, those of skill in the art may choose and design

stated integer or group of integers but not the exclusion of any other integer or group of integers.

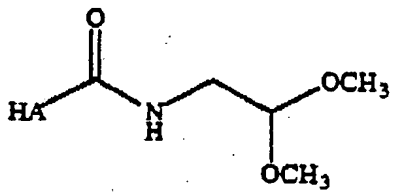
In order that this invention be more fully understood, the following examples are set forth. These examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention in any way.

Example 1

Preparation of N-(2,2-Dimethoxyethyl)-4-(methoxycarbonyl)butanamide (1) - EDC (4.98g, 0.026mol) was added to a mixture of aminoacetaldehyde dimethyl acetal (2.18ml, 20mmol) and methyl monoester of succinic acid (2.64g, 20mmol) in 75ml of dichloromethane, and the reaction mixture stirred for 24 h at room temperature. The solution was extracted successively with 50ml each of ice cold solutions of 0.75M sulfuric acid, 1M NaCl, saturated sodium bicarbonate, and 1M NaCl. The organic phase was collected and dried with sodium sulfate. The solvent was evaporated under reduced pressure yielding a syrup, which showed a single spot on charring upon TLC in solvent A (R_f 0.75) and solvent B (R_f 0.24). The apparent yield of **1** was 65%: ^{1}H NMR in $CDCl_3$ δ 5.70 (bs, 1H, N*H*), 4.34 [t, 1H, C*H*-(OCH_3), 3.67 (s, 3H, $COOCH_3$), 3.43-3.35 (s and t, 8H, C*H*$_3$OC and CHC*H*$_2$NH), 2.38-2.26 (m, 4H, C*H*$_2$CO).

Formation of Acyl-hydrazide (2) *from* **1** - Anhydrous hydrazine (248µl, 7.9mmol) was added to a solution of **1** (1.73g, 7.9mmol) in 5ml of anhydrous methanol. The mixture was stirred at room temperature overnight and the solvent subsequently evaporated under reduced pressure yielding a solid residue. The residue was dissolved in H_2O (6ml) and extracted three times with an equal volume of dichloromethane. The aqueous solution was evaporated to dryness under reduced pressure and then further dried overnight in vacuo. The crystalline solid (1.04g, 82% yield) was homogeneous on TLC in solvent A (R_f 0.10) when visualized by charring. The ^{1}H NMR spectrum indicated the loss of the ester methoxy group when compared to **1**.

(XX)



Example 3

Deprotection of HA-acetals to form HA-aldehydes - The acetal modified HA (formula XXI) was dissolved in H_2O to a concentration of 5-10mg/ml and 1M HCl was added to give a final concentration of 0.025M. The solution was then allowed to stand at room temperature for 0.5 to 1.0h. The solution was neutralized by the addition of 1M NaOH, yielding the deprotected HA-aldehyde (formula XXII).

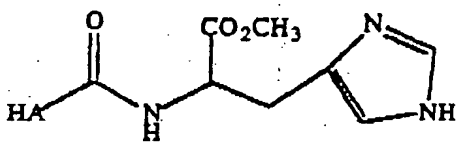
HA-CO-R-CH(OCH_3)$_2$ (XXI) -----> HA-CO-R-CHO (XXII)

Example 4

Preparation of Diaminoethane-HA (formula XXIII) - Sodium hyaluronate (100mg, 0.25mmol) and 1,2-diaminoethane HCl (0.998g, 7.5mmol) was dissolved in H_2O (40ml, 2.5mg/ml HA). The pH was adjusted to 6.5 and HOBT (169mg, 1.25mmol) predissolved in a 1:1 mixture of water and DMSO (1ml) and EDC (240mg, 1.25mmol) was added and the reaction mixture was stirred overnight. The pH was subsequently adjusted to 7.0 with 1M NaOH and NaCl added to produce a 5% w/v solution. HA was precipitated by addition of three volume equivalents of ethanol. The precipitate was redissolved in H_2O at a concentration of approximately 5mg/ml and the precipitation repeated twice. The purified product was freeze dried and kept at 4°C under N_2.

mixture of H_2O and DMSO (1ml) and EDC (240mg, 1.25mmol) was added and the reaction mixture was stirred overnight. The pH was subsequently adjusted to 7.0 with 1M NaOH and NaCl added to produce a 5% w/v solution. HA was precipitated by addition of three volume equivalents of ethanol. The precipitate was redissolved in H_2O at a concentration of approximately 5mg/ml and the precipitation repeated twice. The purified product was freeze dried and kept at 4°C under N_2. See Fig. 3B for NMR data of the product.

(XXV)



Example 7

Preparation of Hydrazido-HA (formula XXVI) - Sodium hyaluronate (100mg, 0.25mmol) and dihydrazide *i.e.* adipic dihydrazide (1.31g, 7.5mmol) was dissolved in H_2O (40ml, 2.5mg/ml HA). The pH was adjusted to 6.5 and HOBT (169mg, 1.25mmol) predissolved in a 1:1 mixture of water and DMSO (1ml) and EDC (240mg, 1.25mmol) was added and the reaction mixture was stirred overnight. The pH was subsequently adjusted to 7.0 with 1M NaOH and NaCl added to produce a 5% w/v solution. HA was precipitated by addition of three volume equivalents of ethanol. The precipitate was redissolved in H_2O at a concentration of approximately 5mg/ml and the precipitation repeated twice. The purified product was freeze dried and kept at 4°C under N_2. See Fig. 3D for NMR data of the product.

molar equivalent of functional groups) is dissolved in a second 1 ml syringe in 1/10 of the HA derivative volume immediately prior to use. The syringes are connected while paying special attention to excluded air, the contents are rapidly mixed, typically with 20 passages, and then extruded. When reacting HA derivative molecules with different functionalities, 0.5-1.0 equivalent of HA-aldehyde is mixed with equivalent of HA-hydrazine, depending on the degree of modification of HA derivatives. At room temperature, gelation occurs within about 30 seconds to several minutes, depending on the formulation, and the gel properties do not significantly change after approximately 5 minutes.

Example 10

Digestion of crosslinked HA hydrogels with hyaluronidase - The general procedure for digestion of crosslinked HA hydrogels is as follows: HA hydrogels are formed in 1ml syringes by crosslinking 12mg/ml HA-amine in phosphate buffered saline with various crosslinkers as indicated in Fig. 4. Gelling is allowed to occur for 1 hour at 37°C for the reaction to be complete, after which identical ~100µl cylindrical gels are formed by cutting the syringes with a razor blade. The gels are incubated with different concentrations of bovine testicular hyaluronidase (Sigma) 50-5000U/mL in 400µl of 30mM citric acid, 150mM Na_2HPO_4, pH 6.3, 150mM NaCl for the indicated time 0-48 hours. Degradation of the gels was determined from the release of glucuronic acid into the supernatant as measured using the carbazole method (Bitter and Muir, Supra).

Example 11

Crosslinked HA hydrogels as a matrix for cell culture -

Chondrocytes were isolated from bovine nose cartilage according to established procedures (Häuselmann et al., Matrix 12, 116-129 (1992; Küttner et al., J. Cell Biol. 93, 743-750 (1982)), cultured in Ham's F12 medium containing 5% fetal bovine serum and antibiotics, and dedifferentiated by monolayer culture on plastic. For cytotoxicity studies, cells ($2.5x10^5$) were embedded into the HA hydrogels by

identical ~3mm thick cylindrical gels were prepared by cutting the syringes with a razor blade. HA hydrogel discs were then placed in each pocket and the skin incisions closed with sutures. Ten days post operatively, the rats were euthanized and the appearance of the implant sites, *i.e.* degree of inflammation, grossly examined and tissue specimens harvested and processed for histology by fixation in phosphate buffered formalin and paraffin embedding. Sections were stained with Haematoxylin/Eosin and with Safranin-O/fast green, and cell infiltration and transformation (cartilage and bone formation) induced by the biomaterial as well as signs of fibrosis and inflammation in the surrounding tissue evaluated. See Figs. 6-8.

2. The composition of claim 1, wherein at least 5% of said disaccharide subunits are substituted disaccharide subunits.

3. The composition of claim 1, wherein at most 95% of said disaccharide subunits are substituted disaccharide subunits.

4. The composition of claim 1, wherein at most 5% of said disaccharide subunits are substituted disaccharide subunits.

5. The composition of claim 1, wherein at least one of said terminal functional groups is selected from the group consisting of peptide, aldehyde, amine, arylazide, hydrazide, maleimide, sulfhydryl, and active ester, whereby said composition is amenable to crosslinking.

6. The composition of claim 1, wherein the molecular weight of said composition is at least 100,000 daltons.

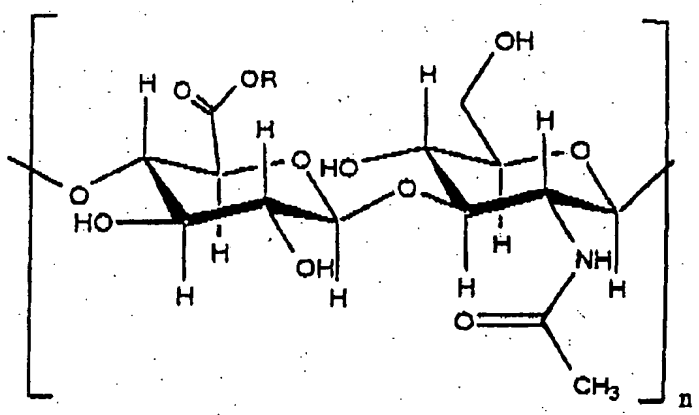
7. The composition of claim 1, wherein the molecular weight of said composition is at most 100,000 daltons.

8. The composition of claim 1, wherein the molecular weight of said composition is at least 1,000,000 daltons.

9. The composition of claim 1, wherein said composition is water soluble.

10. A composition comprising an activated ester of hyaluronic acid of the formula:

(II)



19. A method for forming a matrix for a temporary scaffold for tissue repair according to the method of claim 18, wherein the crosslinker is selected from the group consisting of polyvalent active ester, aldehyde, amine, arylazide, maleimide, and sulfhydryl.

20. A method for forming a matrix for a temporary scaffold for tissue repair according to the method of claim 18, wherein the HA derivative comprises a peptide substrate for transglutaminase, and wherein the HA derivative is crosslinked using transglutaminase.

21. The method of claim 18, wherein step (c) is performed in the presence of cells.

22. The method of claim 18, wherein step (c) is performed in the presence of at least one member selected from the group consisting of growth factors, cytokines, drugs, and bioactive peptides.

23. The method of claim 22, wherein the bioactive peptide is RGD.

24. The method of claim 22, wherein the bioactive peptide is a substrate for transglutaminase.

25. The method of claim 24, wherein the bioactive peptide is APQQEA.

26. The method of claim 24, wherein the growth factor is TGF-β or BMP.

27. The method of claim 18, wherein step (c) is performed *in situ* in a patient in need of tissue repair.

28. A tissue adhesive comprising a hydrogel of claim 11, wherein the side chain is selected from the group consisting of activated ester, aldehyde, arylazide, and maleimide.

29. A tissue adhesive comprising an HA derivative of claim 10.

30. A tissue adhesive comprising a hydrogel of claim 11, wherein the crosslinked HA derivatives are formed using a cross-linker selected from the group consisting of polyvalent active ester, aldehyde, arylazide, and maleimide.

Fig. 1

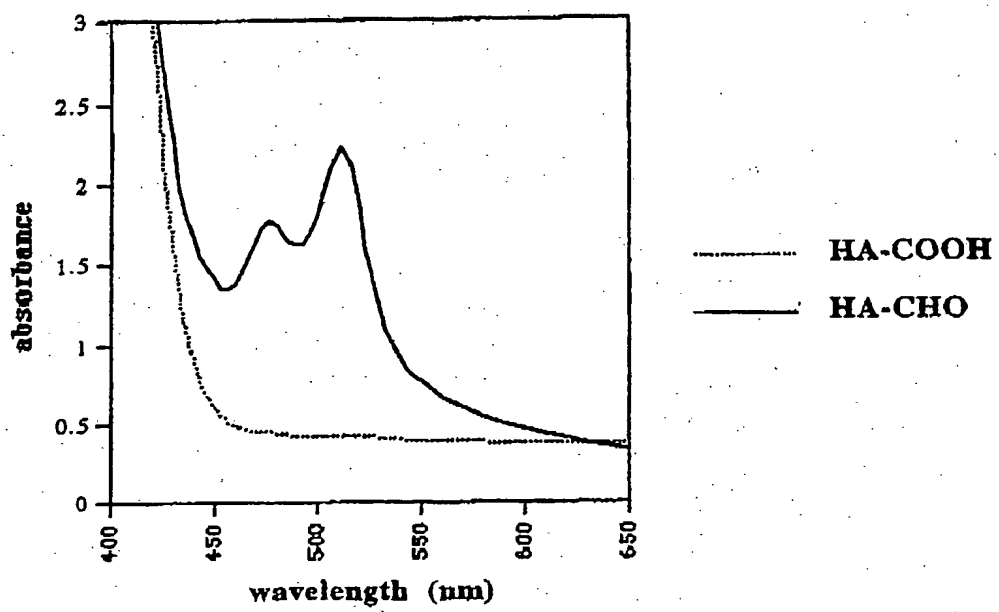


Fig. 2B

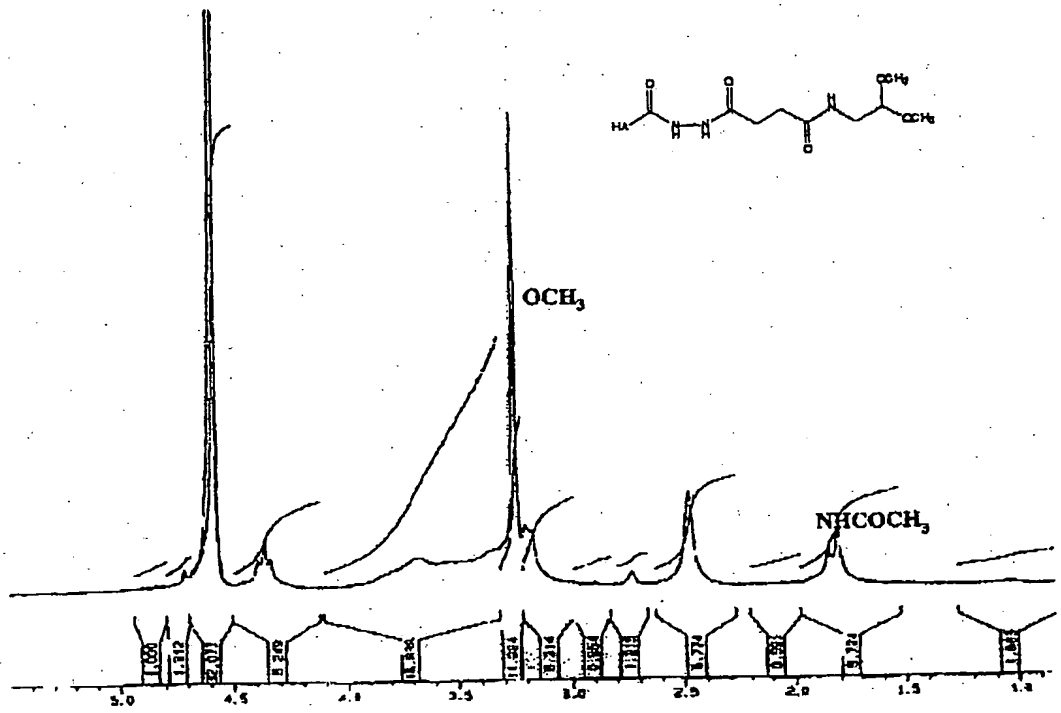


Fig. 3B

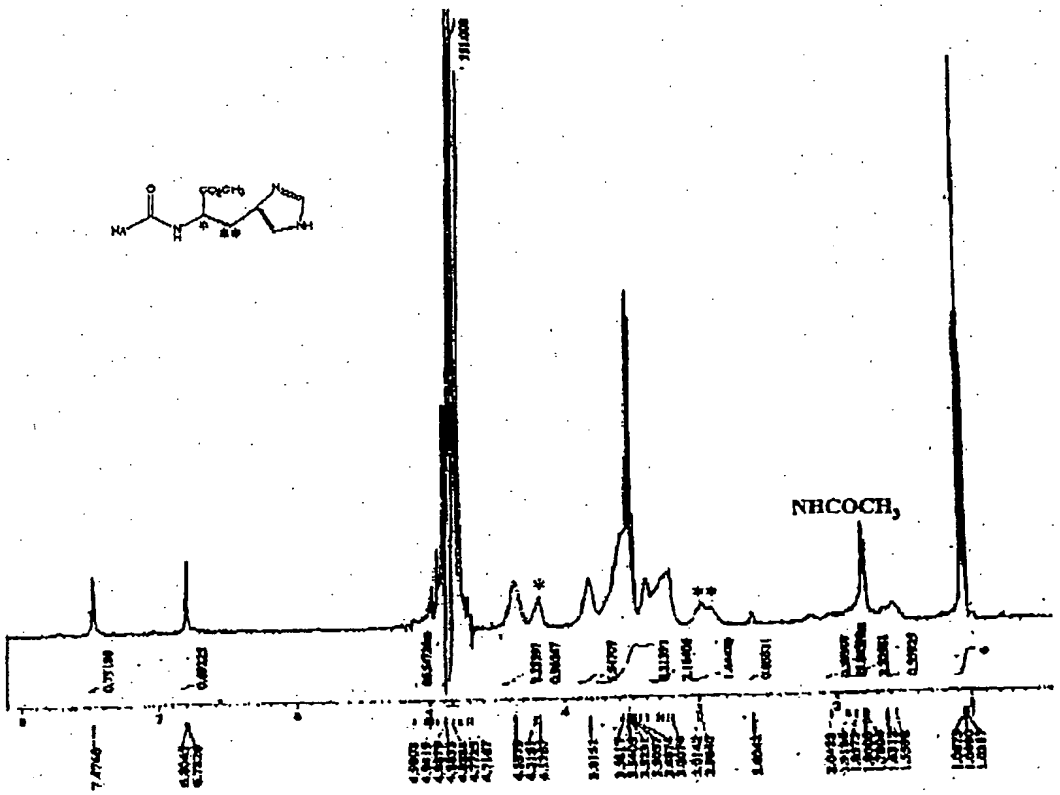


Fig. 3D

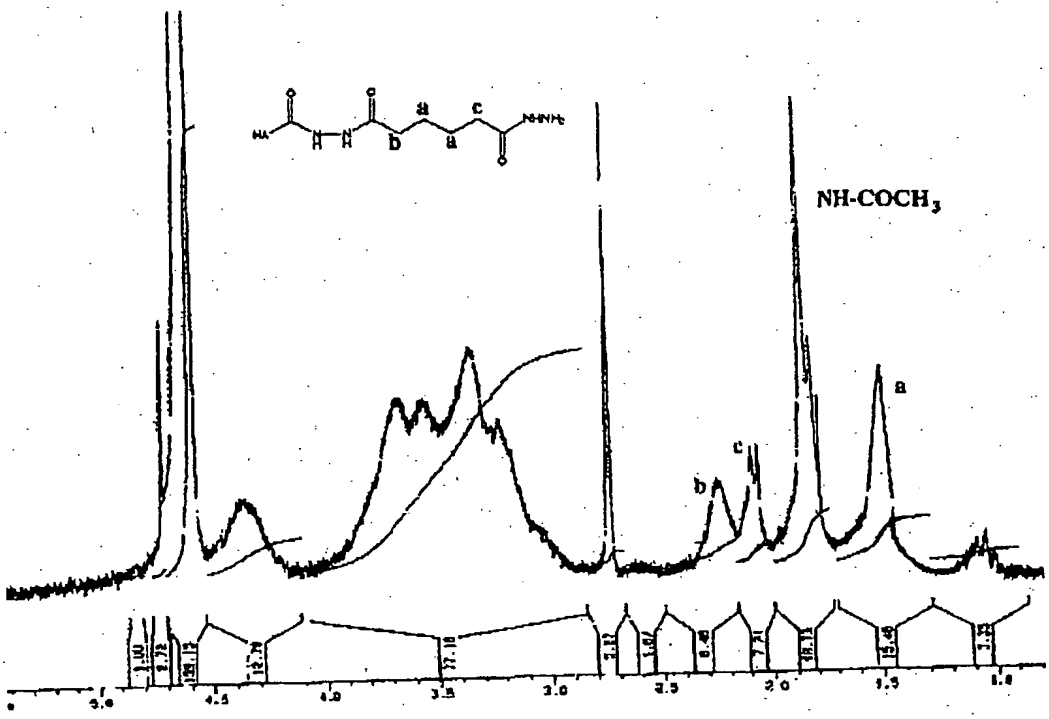


FIG. 4B

B

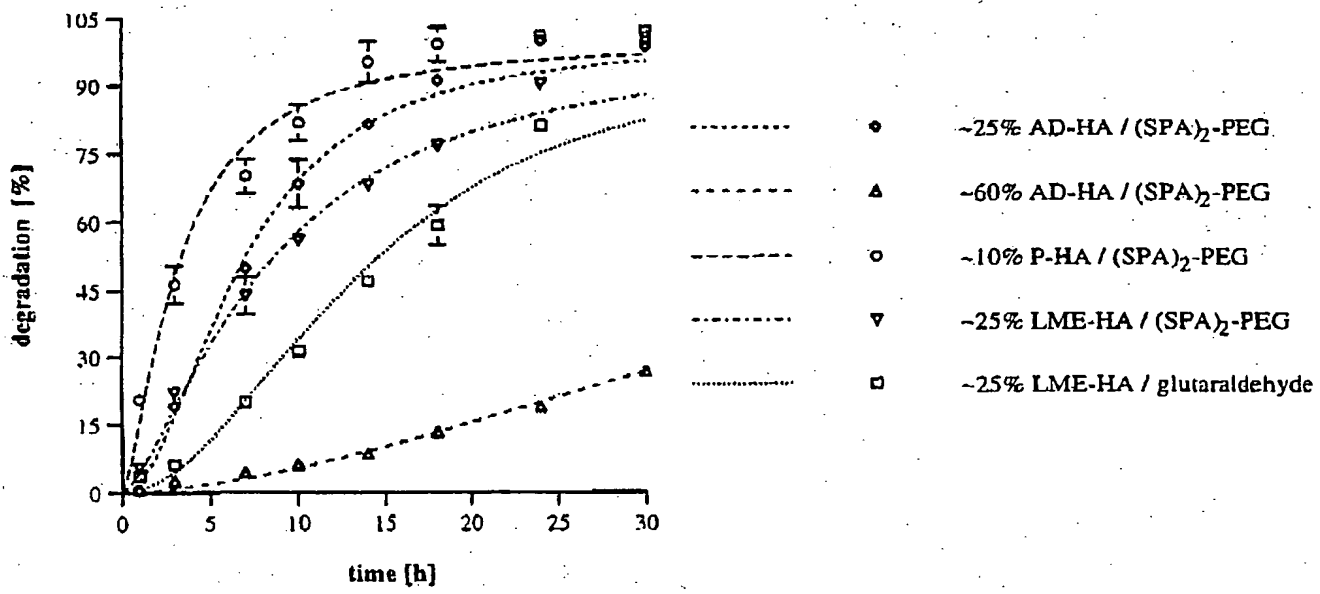

degradation [%]
105
90
75
60
45
30
15
0
0
5
10
15
20
25
30
time [h]
~25% AD-HA / (SPA)2-PEG
~60% AD-HA / (SPA)2-PEG
~10% P-HA / (SPA)2-PEG
~25% LME-HA / (SPA)2-PEG
~25% LME-HA / glutaraldehyde

Fig. 6

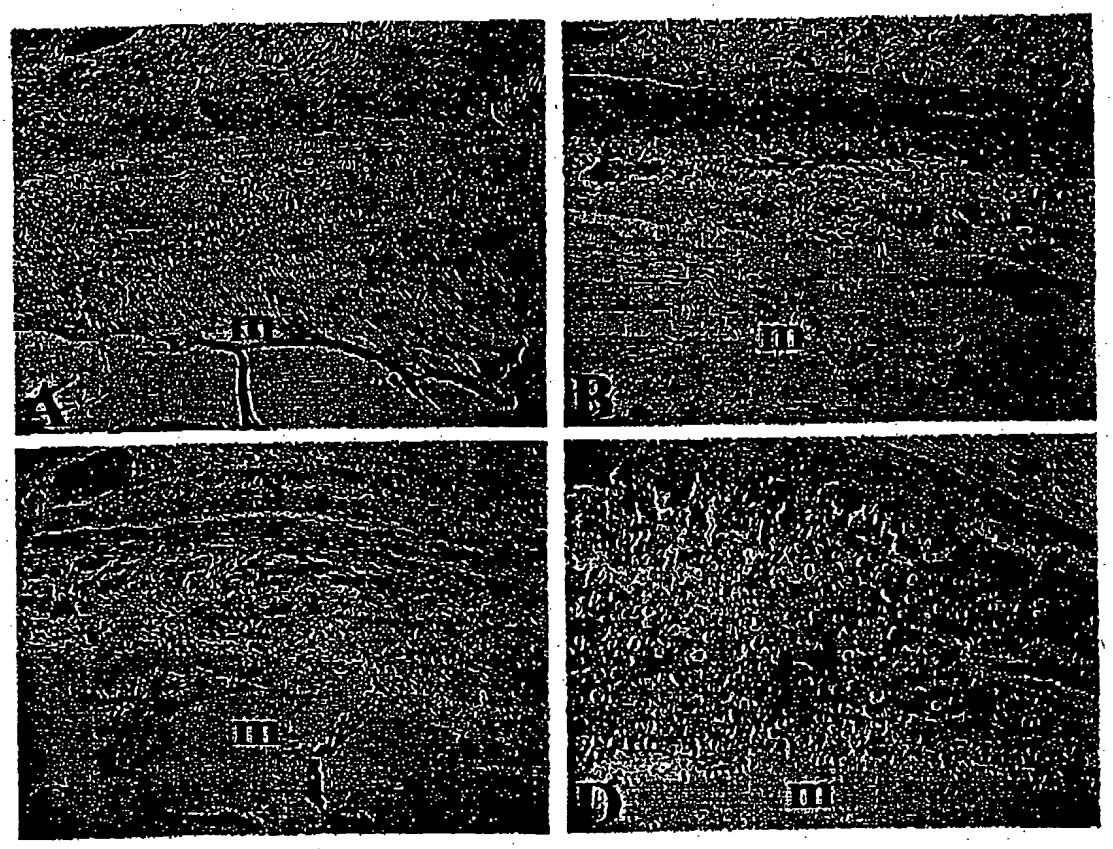
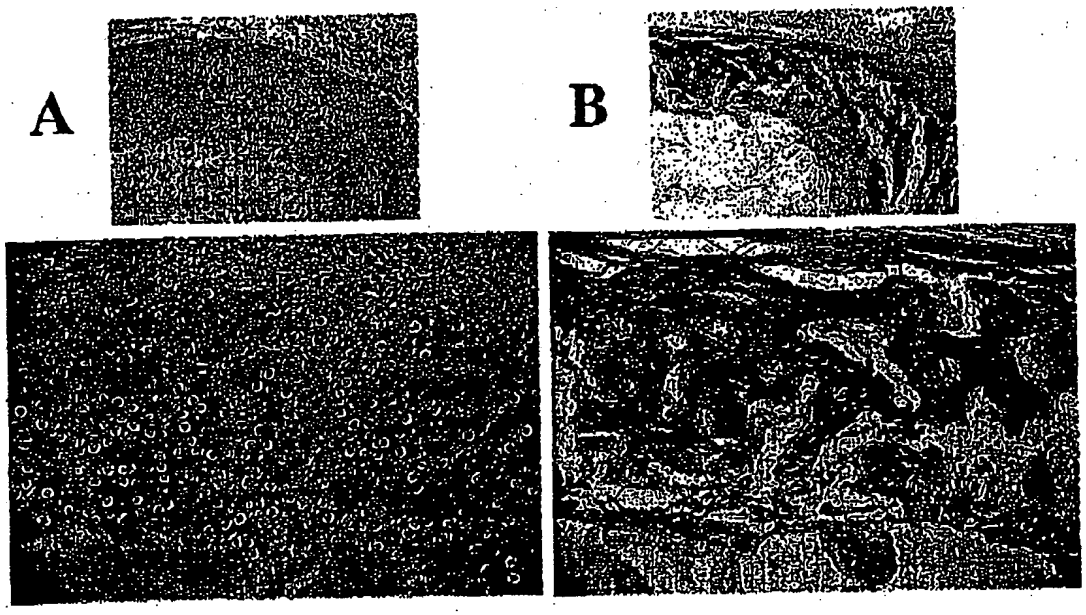


Fig. 8



INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/EP 99/06913

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passagee	Relevant to claim No.
X	US 5 527 893 A (BURNS JAMES W ET AL) 18 June 1996 (1996-06-18) column 14, line 14-33; example 1	1,6,8, 11,13
A	US 5 652 347 A (POUYANI TARA ET AL) 29 July 1997 (1997-07-29) cited in the application column 3, line 6-16; claims 1-3,8; examples 1-5	
A	WO 97 18244 A (SEIKAGAKU KOGYO CO LTD ;WAKI MICHINORI (JP); MIYAMOTO KENJI (JP)) 22 May 1997 (1997-05-22) page 13, line 15-25 page 16, line 15,16; claims 1-5	
A	WO 90 06767 A (JOLLA CANCER RES FOUND) 28 June 1990 (1990-06-28) page 14, line 1 -page 15, line 4; claims 1,6; examples II,V	

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